

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner  
 US Department of Commerce  
 United States Patent and Trademark  
 Office, PCT  
 2011 South Clark Place Room  
 CP2/5C24  
 Arlington, VA 22202  
 ETATS-UNIS D'AMERIQUE  
 in its capacity as elected Office

<b>Date of mailing</b> (day/month/year) 02 November 2000 (02.11.00)	
<b>International application No.</b> PCT/GB00/00761	<b>Applicant's or agent's file reference</b> NW/7125INT
<b>International filing date</b> (day/month/year) 03 March 2000 (03.03.00)	<b>Priority date</b> (day/month/year) 05 March 1999 (05.03.99)
<b>Applicant</b> ARMOUR, John, Anthony	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:  
 29 September 2000 (29.09.00)

☐ in a notice effecting later election filed with the International Bureau on:  
 \_\_\_\_\_

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	<b>Authorized officer</b> Juan Cruz Telephone No.: (41-22) 338.83.38
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# TENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>NW/7125INT</b>	<b>FOR FURTHER ACTION</b> <small>see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.</small>	
International application No. <b>PCT/GB 00/ 00761</b>	International filing date (day/month/year) <b>03/03/2000</b>	(Earliest) Priority Date (day/month/year) <b>05/03/1999</b>
Applicant  <b>THE UNIVERSITY OF NOTTINGHAM et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of Invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 26 and partially 23-25.

Claims 23-26 do not meet the requirements of Article 6 PCT. The subject matter for which protection is sought in claims 23-26 is imprecisely defined. Claims 23-25 make reference in their formulations to subject matter in "the accompanying examples and drawings" without specifying which examples or drawings are precisely being referred to. Thus, for example, although a method and the probes for use used in the method may be determined from fig. 1, no similar information as to either can be extracted from figs 2-6. In consequence, the subjected matter searched in claims 23-25 were restricted to the following: Claim 23: to the method defined in claim 1 and pictorially represented in fig.1; Claims 24 and 25: to the probes and a Kit for performing the method of claim 1 and pictorially represented in Fig. 1. No subject matter is defined in the formulation of claim 26. Consequently, this claim was not searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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JUN 22 2001

PCT

Applicant's or agent's file reference NW/7125INT	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB00/00761	International filing date (day/month/year) 03/03/2000	Priority date (day/month/year) 05/03/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant THE UNIVERSITY OF NOTTINGHAM et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 9 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  29/09/2000	Date of completion of this report  20.06.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Giry, M  Telephone No. +49 89 2399 7328 

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/00761

**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-12 as originally filed

**Claims, No.:**

1-26 as originally filed

**Drawings, sheets:**

1/6-6/6 as originally filed

**Sequence listing part of the description, pages:**

1-2, filed with the letter of 25.05.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00761

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

### III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 23-26.

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 23-25 are so unclear that no meaningful opinion could be formed (*specify*):  
**see separate sheet**
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 26.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

### V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00761

## 1. Statement

Novelty (N)	Yes:	Claims	1-20, 22
	No:	Claims	21
Inventive step (IS)	Yes:	Claims	1-20, 22
	No:	Claims	21
Industrial applicability (IA)	Yes:	Claims	1-22
	No:	Claims	

## 2. Citations and explanations see separate sheet

## VI. Certain documents cited

### 1. Certain published documents (Rule 70.10)

and / or

### 2. Non-written disclosures (Rule 70.9)

see separate sheet

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/GB00/00761

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. Claim 26 was not searched and thus will not be examined (Rule 66.1(e) PCT).
2. The subject-matter of claims 23-25 is not characterized by any technical feature. Consequently, the subject-matter of said claims is so unclear that it is impossible to compare the claimed subject-matter with the prior art. A meaningful examination of novelty and inventive step is not possible.

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1 - **Reference** is made to the following documents :

D1 : WO 95 25538, 28 September 1995

D2 : WO 93 04199 A, 4 March 1993

D3 : WO 90 06042 A, 14 June 1990

2 - **Novelty** - Art. 33(1) and (2) PCT :

Notwithstanding the clarity objection (see point VIII-2), claims 21 and 22 will be examined taking into account the features of the probes given in claim 1.

- 2.1 Document D1 discloses a method for identifying a polymorphic restriction site in nucleic acid comprising (a) digesting DNA with a first restriction endonuclease, (b) ligating to the ends of the reaction products of step (a) a first adaptor, (c) digesting the products of step (b) with a second restriction endonuclease, (d) ligating to each of the ends of the reaction products generated in step (c) a second adaptor. The products thus obtained constitute a set of probes flanked by primer binding



sites amplifiable by PCR using primers complementary to these adaptor sequences (p. 101, claim 50 ; Figures 9A, 9B and 9C). Document D1 thus appears to be novelty destroying for claim 21.

- 2.2 None of the available prior art documents disclose a method of screening for copy number of target nucleic acid sequences using a set of probes characterized by the same primer binding sites flanking the sequences complementary to the target sequences. Therefore, the method to which claims 1-20 relate and the kit for realizing said method, subject-matter of claim 22 can be considered as novel.

**3 - Inventive step - Art. 33(1) and (3) PCT :**

- 3.1 Document D2 discloses a method for establishing the presence of a nucleic acid in a sample comprising the steps of (a) exposing the sample containing the putative target sequence to an immobilized nucleic acid probe-primer characterized by a complementary portion enabling hybridization of the target sequence ; (b)(i) subjecting any probe-primer/target hybrids to a PCR so as to extend and label the probe-primer ; (b)(ii) subjecting any probe-primer/target hybrids so formed to hybridization of a portion of the target sequence adjacent to the previously hybridized target sequence with a second labelled oligonucleotide primer complementary to the sequence of said adjacent portion, (b)(iii) ligating said probe-primer and second primer ; (c) detecting or quantitating any label bound to the extended immobilised probe-primer (p. 4, line 34 to p. 5, line 25 ; p. 37, claim 1).
- 3.2 Document D3 discloses a method for the quantitative determination of target RNA or DNA in an analyte sample comprising the steps of (a) contacting the analyte with magnetic particles carrying a complementary DNA probe ; (b)(i) subjecting to (RT)-PCR conditions in the presence of labelled nucleotides using the probe as a primer whereby labelled complementary DNA is formed if said RNA or DNA was present in said analyte ; (b)(ii) magnetically aggregating the magnetic particles and removing PCR reagents ; (c) detecting and determining the amount of said label (p. 3, lines 20-35).

3.3 Independent claim 1 refers to a method of determining the copy number of target nucleic acid sequences in a sample comprising the steps of (a) denaturing and immobilizing test DNA sample ; (b)(i) hybridizing a set of amplifiable probes characterized by the same primer binding sites flanking the sequence complementary to the target sequences ; (b)(ii) stringent washing allowing retention only of specifically bound probes ; (b)(iii) recovering the probes ; (b)(iv) subjecting the probes to PCR amplification using a common primer pair ; (c) determining quantitatively the copy number of the nucleic acid sequences present in the sample by analysis of the respective amounts of amplified probes.

3.4 In the light of the prior art documents dealing with quantitative PCR procedures, the method of the invention differs by the use of a set of amplifiable probes characterized by the same primer binding sites flanking the sequences complementary to the target sequences, that can be recovered quantitatively after hybridization. The same primer pair binding sites flanking all the different probes allows the different bound probes to be amplified together with a single primer pair and thus the analysis of a plurality of different nucleic acid sequences simultaneously. Therefore, the determination of the quantity of each probe product enables quantitative determination of the copy number of the respective sequences in the sample. The problem to be solved by the present invention can therefore be seen in providing an alternative quantitative PCR methodology.

Since there is no hint in the prior art to arrive at the solution proposed by claims 1-20, and that this solution cannot be deduced in an obvious way from the teaching in the available prior art documents, either if taken alone or in any combination, claims 1-20 meet the requirements of the PCT with respect to inventive step.

3.5 Insofar as the set of probes it contains is limited to a set as defined in claim 1 only, an opinion can be given as regards inventive step of the kit subject-matter of independent claim 22. Said kit can also be regarded as inventive since the reagents it contains allow to perform the inventive method of the invention.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/00761

**Re Item VI**

**Certain documents cited**

**Certain published documents (Rule 70.10)**

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO 00/43538	27.07.00	19.01.00	19.01.99

Should the present application enter the national or regional phase, the above cited document which discloses amplifiable oligonucleotide probes comprising a central segment of randomly varied bases flanked on each side by segments of a defined sequence (p. 22, claim 1 ; Figure 1), could be relevant for the question of novelty.

**Re Item VIII**

**Certain observations on the international application**

1. Since the inventive concept of the method lies on the amplification of the probes, it appears that a step allowing the separation of the bound probes from the unspecific unbound probes is necessary to allow the amplification of specific sequences complementary to the target sequences. Therefore, the step of immobilization of the sample prior to hybridization to the probes and of recovering the bound probes prior to amplification, as described in description on p. 7, line 21 to p. 8, line 13 and on Figure 1, and presented as a preferred embodiment on p. 2, lines 23-25, is a step essential to the method of the invention. Since independent claim 1 does not contain these features it does not meet the requirement following from Art. 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention.
2. Claims 21 and 22 lack clarity since the set of probes to which they relate is not characterized by any technical feature (Art. 6 PCT).

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/GB00/00761

3. In claim 2, the feature mentioned after "for example" is regarded as an optional feature which has no limiting effects on the claim (Art. 6 PCT).
4. Claim 4 does not meet the requirements of Art. 6 PCT in that the matter for which protection is sought is not clearly defined. The claim attempts to define the probes in terms of the result to be achieved which merely amounts to a statement of the underlying problem.
5. Claims 15 and 22 do not meet the requirements of Art. 6 PCT in that the matter for which protection is sought is not clearly defined. The technical features necessary for defining "means to obviate or mitigate hybridization" and "means to enable amplification and analysis of amplification products" are missing.
6. Claims 23-25 do not meet the requirements of Art. 6 PCT because they do not contain any technical feature (Art. 6 PCT).

# INTERNATIONAL SEARCH REPORT

International Application No

GB 00/00761

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ARMOUR ET AL: "MEASUREMENT OF LOCUS COPY NUMBER BY HYBRIDISATION WITH AMPLIFIABLE PROBES" NUCLEIC ACIDS RESEARCH, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 28, no. 2, 2000, pages 605-609, XP002138423 ISSN: 0305-1048 the whole document	1-22
A	WO 93 04199 A (SCIENT GENERICS LTD) 4 March 1993 (1993-03-04) claim 1	1,21
A	WO 90 06042 A (HOLMES MICHAEL JOHN ; DYNAL AS (NO)) 14 June 1990 (1990-06-14) claim 1	1,21



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

29 September 2000

Date of mailing of the international search report

06/10/2000

Name and mailing address of the ISA

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Osborne, H

## INTERNATIONAL SEARCH REPORT

International Application No

P GB 00/00761

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 07892 A (UNIV CALIFORNIA) 18 February 1999 (1999-02-18) page 2, line 19 -page 3, line 2	1
E	WO 00 43538 A (UNIV MONTREAL ;BRUKNER IVAN (CA); PAQUIN BRUNO (CA); TREMBLAY GUY) 27 July 2000 (2000-07-27) figure 1	21
A	WO 98 15649 A (CREMER THOMAS ;CRAIG JEFF (DE); UNIV HEIDELBERG (DE)) 16 April 1998 (1998-04-16) figure 1	21
A	HILL J M ET AL: "QUANTITATIVE ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCTS BY DOT BLOT" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 235, no. 1, 1 March 1996 (1996-03-01), pages 44-48, XP000552019 ISSN: 0003-2697 the whole document	1
A	REISCHL U ET AL: "QUANTITATIVE PCR A SURVEY OF THE PRESENT TECHNOLOGY" MOLECULAR BIOTECHNOLOGY,US,TOTOWA, NJ, vol. 3, 1995, pages 55-71, XP000600241 ISSN: 1073-6085 the whole document	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

P/GB 00/00761

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9304199	A	04-03-1993	NONE	
WO 9006042	A	14-06-1990	AT 102256 T	15-03-1994
			AT 93896 T	15-09-1993
			AU 640626 B	02-09-1993
			AU 4758690 A	26-06-1990
			AU 634993 B	11-03-1993
			AU 4759690 A	26-06-1990
			CA 2003501 A	21-05-1990
			CA 2003508 A	21-05-1990
			DE 68908900 D	07-10-1993
			DE 68908900 T	20-01-1994
			DE 68913555 D	07-04-1994
			DE 68913555 T	07-07-1994
			WO 9006045 A	14-06-1990
			EP 0446260 A	18-09-1991
			EP 0444120 A	04-09-1991
			JP 4501956 T	09-04-1992
			JP 3020271 B	15-03-2000
			JP 4501959 T	09-04-1992
			US 5512439 A	30-04-1996
WO 9907892	A	18-02-1999	US 6066453 A	23-05-2000
			EP 1012332 A	28-06-2000
WO 0043538	A	27-07-2000	NONE	
WO 9815649	A	16-04-1998	AU 4116397 A	05-05-1998
			EP 0951566 A	27-10-1999

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12Q 1/68</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/53804</b> <b>(43) International Publication Date:</b> 14 September 2000 (14.09.00)
<b>(21) International Application Number:</b> PCT/GB00/00761 <b>(22) International Filing Date:</b> 3 March 2000 (03.03.00) <b>(30) Priority Data:</b> 9904991.8      5 March 1999 (05.03.99)      GB <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF NOTTINGHAM [GB/GB]; University Park, Nottingham NG7 2RD (GB). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> ARMOUR, John, Anthony [GB/GB]; The University of Nottingham, University Park, Nottingham NG7 2RD (GB). <b>(74) Agent:</b> WOMSLEY, Nicholas; Swindell & Pearson, 48 Friar Gate, Derby DE1 1GY (GB).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> GENETIC SCREENING  <b>(57) Abstract</b>  A method of screening for nucleic acid sequence copy number in a sample genetic material (10), the method including introducing a number of different genetic probes (14) to hybridise with the genetic sample (10). The probes (14) are flanked by the same or substantially the same primer binding sites thereby enabling amplification of sample-bound probes using a single primer pair and thus the screening of different sequence copy numbers at the same time.		



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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### Genetic Screening

The present invention relates to screening for sequence copy number and in particular to screening for changes in copy number of a plurality of nucleic acid sequences substantially simultaneously.

The loss or reduction in the normal number of copies of a genetic sequence (deletion) or the increase in copy number (amplification) are of widespread general importance. Such genetic alterations are known to underlie phenotype characteristics both somatic and germline including disorders as diverse as idiopathic mental retardation and neoplasia. The demonstration of the site and nature of such genetic alteration is critical in the identification of the genes responsible and to the development of appropriate and effective treatments and therapies.

In principle, it is possible to screen for genetic deletions by using Southern blot hybridisation. However, this method requires heterozygosity, and whilst the loss of heterozygosity at polymorphic loci can be used to demonstrate the absence of one allele in the soma or in the germline, its effectiveness is limited by the requirement for heterozygosity and the number of repeated tests needed to screen even a small fraction of the genome.

If sufficient precautions are taken to assure quantitative yields, the polymerase chain reaction (PCR) can be used to screen for copy number, and specialised systems have been developed to assure quantitative PCR by following the accumulation of products as amplification proceeds. However, such systems do not provide for the satisfactory analysis of a plurality of alterations substantially simultaneously.

According to the present invention there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target

sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to their respective sequences, and amplification of sample-bound probes using a pair of primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample.

Preferably each probe is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel. Preferably the plurality of different probes comprises a predetermined set of different probes each chosen to be specific for a respective target nucleic acid sequence. The set may comprise probes suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced, preferably using the polymerase chain reaction (PCR), enables quantitative determination of the copy number of the respective sequences in the sample. The method may be used to screen sequences of different genes or different sequences within a gene, such as different exons in a eukaryotic gene. Preferably the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).

Preferably the genetic material is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised. Preferably an excess of probes is used.

Probes labelled for ready identification, such as with fluorescent labels are preferably used. More than one set of probes may be used, either simultaneously or sequentially. The flanking primer pairs may be the same or different for each set of probes.

Preferably the method comprises means to obviate or mitigate hybridisation between primer binding sequences. Competing oligonucleotides

may be introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.

Preferably unbound probes and primers are thoroughly washed away from the bound probes following hybridisation stage and prior to analysis.

The method may be used to screen DNA, RNA and/or cDNA with appropriate probe sets. The method may be used to screen somatic and/or germline sequences. The method may be used to screen for polymorphic alterations.

The invention further provides a set of probes substantially as described above in any of the preceding seven paragraphs.

The invention may still further comprise a kit for such a method described above in any of the preceding eight paragraphs, which kit comprises a probe set generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).

The present invention will now be described by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 is a simple schematic illustration of a method according to the invention;

Fig. 2 shows a gel run produced from samples from four unrelated humans using the method according to the present invention;

Fig. 3 shows an enlargement of the central region III of the gel in Fig. 2;

Fig. 4 shows the results of quantitative analysis of the region III of the gel from one male sample (upper trace) and one female (lower trace);

Fig. 5 shows the relative signal from different probes shown plotted against probe size;

Fig. 6 shows the *EcoRV* cloning site of pZero2 and the corresponding

primer combination (PZA and PZB) used to generate probes from fragments cloned into the *EcoRV* site.

With reference to Fig. 1, there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to the respective sequences, and amplification of sample-bound probes using a pair of flanking primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acids sequences in the sample.

The method can be used to screen for genetic alterations, in particular deletions and amplifications in copy number of genetic sequences, both somatic and germline.

The sample of genetic material to be screened, which may comprise, DNA, cDNA and/or RNA, shown diagrammatically in Fig. 1 (10), is reversibly immobilised on a medium (12) such as a nylon filter.

A plurality of probes (14) is designed and prepared to seek out and hybridise with specific nucleic acid sequences, if present in the sample. The probes (14) are chosen to comprise a set of different probes each type operable to seek out a different respective nucleic acid sequence which may be sequences suspected of being involved in a particular disorder or condition for example, in the organism from which the sample has been taken. Alterations in the copy number of particular genetic sequences are known to be involved in conditions such as cancer and mental retardation, and probes specific for these sequences can be prepared and comprised in a probe set used in accordance with the present methodology for example where certain symptoms suggest these disorders or diseases may be involved.

When an appropriate probe set is created or identified, it is then introduced to the immobilised genetic material in stoichiometric excess under conditions favouring hybridisation of the probes to their respective nucleic acid sequences, if present.

Once sufficient time has elapsed to provide for hybridisation, unbound probes are washed thoroughly away and the PCR employed to amplify bound probes.

The use of the same primer pair binding sites flanking all the different probes is important to the successful operation of this method, and allows different bound probes to be amplified together with a single primer pair and thus the screening for a plurality of different loci at the same time.

Some interaction has been experienced between primer binding sites flanking different probes, but this problem has been addressed by adding to the hybridisation solution an excess of competing oligonucleotides which releasably bind to the respective primer binding sites thereby preventing such undesirable interactions whilst not adversely affecting the subsequent amplification process. The oligonucleotides are displaced once hybridisation of the probes to the sample is complete, to provide for hybridisation of the amplification primers to the binding sites.

Respective sets of probes can be made either by ligation to oligonucleotide adapters, or more simply by preparing the probes as inserts into the same site of a cloning vector, and using primers flanking the cloning site for amplification. Fig. 6 shows the cloning site of the vector pZero2 (sequence provided by InVitrogen: <http://www.invitrogen.com/vecseq-gcg/pzero2.seq>), showing the primers PZA and PZB flanking the *EcoRV* site (lowercase) used to clone blunt-ended fragments. Products amplified using these primers are therefore 59bp longer than the cloned inserts. It is thus possible to prepare sets of probes, each detecting a different locus, but all flanked by the same primer-binding sites.

It is important that a probe set is created such that each contributing probe is distinguishable, for example by virtue of its mobility through a separating gel. Following amplification, the appearance of a product of corresponding mobility acts to report the presence of a particular nucleotide sequence, and hence the presence of that sequence in the target sample. As an excess of probe is used, and amplification is restricted to the quantitative phase, the absolute amount of each product reflects its copy-number in the sample tested. Different probes may amplify with different efficiencies, but the proportional contribution from each probe will reflect its dosage in the sample and can be inferred from comparisons between different samples examined with the same probe set. As it is the retained probe that is amplified, not the sample, the gel mobility of the product is not influenced by the presence (for example) of Restriction Fragment Length Polymorphism (RFLPs) at that locus.

The following outlines experiments conducted using the method of the present invention to screen a plurality of different genetic sequences simultaneously. A set of 43 amplifiable probes was generated by cloning blunt-ended fragments into the *EcoRV* site of pZero2 (Fig. 6), followed by amplification with vector primers flanking this site, and selection of a mix of probes of different lengths. This mix included probes from 7 autosomes (1, 5, 11, 16, 17, 18 and 22), as well as the X and Y chromosomes.

The initial mix of 43 probes was subcloned from plasmids and plasmid fragments known from sequencing or hybridisation not to contain dispersed repeat elements; in the case of one of the Y-linked probes (SRY), a 1360bp fragment was amplified using primers SRYA (5'GCAGTAGAGCAGTCAGGGAG3') and SRYB (5'GGGGAGAGAAAGAAACAAGTTTG3'). Other sources of cloned genomic DNA were: chromosome 1, pJBT2 (JALA, unpublished); chromosome 5, pMS621 (Armour et al., 1996); chromosome 11, pEJ6.6 (Goldfarb et al., 1982); chromosome 16, pRN6N6A (JALA and R.M. Badge, unpublished); chromosome 17, pYNZ22 (Nakamura et al., 1988); chromosome 18, pMS440 (Armour et al., 1990); chromosome 22, pMS632c (Armour et al., 1995); chromosome X, pMS613 (Armour et al., 1990). After isolation of the genomic insert and digestion with



frequently-cutting restriction enzymes producing blunt ends (generally double digestion with *AluI* plus *HaeIII*), the resulting smaller fragments were cloned into the *EcoRV* site of pZero2 (InVitrogen) and propagated in *E.coli* TOP10 (InVitrogen). Care was taken to avoid repeat regions in DNA from minisatellite-containing clones.

With reference to Fig. 6, probes prepared by cloning blunt-ended restriction fragments from primary clones into the *EcoRV* site of pZero2 (InVitrogen) were amplified directly from bacterial cells (Sandhu et al., 1989) using flanking vector primers PZA (5'AGTAACGGCCGCCAGTGTGCTG3') and PZB (5'CGAGCGGCCGCCAGTGTGATG3'). The positions of these primers in the pZero-2 cloning site is shown in figure 6. PCR was carried out in Advanced Biotechnologies Buffer IV (75mM Tris-HCl pH8.8, 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween), with 0.2mM each dNTP, 1mM MgCl<sub>2</sub>, 0.2μM each primer and 0.05U/μl Taq DNA polymerase (Advanced Biotechnologies). Reactions (generally 10μl) were subjected to 25 cycles of (95°C for 1 minute/70°C for 1 minute). Products amplified using <sup>33</sup>P end-labelled PZA were separated on denaturing 6% polyacrylamide/50% urea gels and probe mixes assembled such that the mobilities of all constituent probes were distinct.

Sample DNA for immobilisation was prepared in an initial volume of less than 5μl, denatured by addition of 1μl 1M NaOH, and spotted onto a nylon filter (MSI MAGNA, approximate dimensions 2mm x 4mm), 1μl at a time, allowing the solution to dry between applications. When all the DNA had been added the DNA was irreversibly bound to the filter using U.V. irradiation. Since numerous filters were to be used in the same hybridisation, individual marks cut into the edges and corners made clear the identity of each filter.

Filters were prehybridised in 1ml of prehybridisation solution (0.5M sodium phosphate pH7.2, 7% SDS, 0.1mg/ml alkali-denatured herring sperm DNA) at 65°C overnight, and before hybridisation this was exchanged for 200μl of prehybridisation solution with the addition of denatured human Cot-1 DNA (Gibco BRL) to a final concentration of 10μg/ml. Probe mixtures containing

about 100pg of each sequence in 1µl were mixed with 7µg *E.coli* DNA (DH5α DNA, digested with *Hae*III) and 1µg human Cot-1 DNA (Gibco-BRL), and denatured by adding 2µl 1M NaOH. After 1 minute at 37°C, the probe mixture was placed on ice, neutralized by adding 3µl 1M NaH<sub>2</sub>PO<sub>4</sub>, and added to the hybridisation solution.

Hybridisation was left to proceed at 65°C overnight, and the filters were thoroughly washed at 65°C in (a) two 1ml changes of prehybridisation solution, (b) 200ml 1x SSC/1% SDS and (c) 500ml 0.1x SSC 0.1% SDS. Washed filters were then transferred to individual 50µl amplification reactions (constituents as for probe amplification), and bound DNA amplified for 5 cycles of 95°C 1 minute/70°C 1 minute.

This low-level preamplified solution was then used to seed further 10µl amplifications using <sup>33</sup>P 5' end-labelled primer PZA. Labelled PCR products were given a final incubation at 72°C for 20 minutes (to drive to completion the addition of untemplated A at the 3' ends), an equal volume of formamide dye mix (98% formamide, 10mM EDTA pH 8.0, 1mg/ml xylene cyanol FF, 1mg/ml bromophenol blue) added, and the DNA denatured at 100°C for 2 minutes and then placed on ice. From this denatured sample, 4µl was loaded into a well of a 6% polyacrylamide/50% urea gel in 0.5x TBE, and the gel run at 90W (to maintain a gel temperature of about 45°C) until the xylene cyanol dye was close to the end of the gel. Gels were fixed in 10% methanol/10% acetic acid, dried, and radioactivity detected either by standard autoradiography or (for quantitative analysis) using ImageQuant software analysis on data captured by a storage phosphorimager screen (Molecular Dynamics).

The screening results of hybridising this probe set with DNA from one male and three females is shown in figure 2. The autosomal loci give comparable signals, between all individuals; while there is probe-to-probe variation in absolute intensity, the relative contribution of each band to the whole is highly reproducible. By contrast, the Y-derived probes only give strong signals in the hybridisation with male DNA. The relative signal from the X-

linked probes is reduced by about 50% in the male (figures 3 and 4).

In figure 5, the normalised relative signal from each probe is shown plotted against the length of the probe; signal intensities are normalised relative to the mean value for that probe among all individuals tested for autosomal loci, among all the females for X-linked probes and relative to the male for Y-linked probes. The boxed points are the points for X-linked loci in the male (about 50% signal intensity) and for Y-linked probes in females (close to zero). This experiment models the expected detection of homozygous deletions (Y-linked loci in females) or hemizygous deletions (X-linked loci in males). In the size range 140-600bp the signal appears to reflect copy number variation consistently.

Nearly all the amplified bands seen after hybridisation corresponded in size with the expected probes. There were however three bands (marked with asterisks on figure 2) which did not correspond to probes of known origin; the largest of these is clearly Y-linked. The simplest explanation for the appearance of these bands is that they are the result of contamination of the probe mix by other probes made at the same time. Since (if hybridisation has gone to near completion) the intensity of the band recovered from a target DNA will depend on its copy-number in the target DNA, and not on its concentration in the probe mix, even relatively minor contamination with additional probes could result in the appearance of unexpected bands in the amplified product at intensities equal to the predicted bands. Probe fragments corresponding to two of these additional bands are detectable as components of the initial probe mix. The importance of avoiding even minor contamination with additional probes represents an advantage of isolating and storing the probes as clones (rather than PCR products), since uncontaminated probe preparations can be reconstituted from colony-purified clones.

The efficiency of this method derives from its ability to examine numerous loci simultaneously (in this experiment, 43 probes). In the format used here, in which radiolabelled probes are resolved using denaturing

polyacrylamide gel electrophoresis, the number of probes which can be analysed simultaneously is limited only by the resolving power of the gel. Since probes in the size range 140-600bp have been used successfully in this experiment, careful size-selection of probes could lead to probe sets containing more than a hundred probes, which might (for example) be used as chromosome-specific subsets to analyse copy number at megabase resolution.

Moreover, greater resolution may be achieved using fluorescently-labelled probes and apparatus and software designed for automated sequence analysis. This would not only have the advantage of single-base resolution over a larger range (and hence the possibility of more probes per lane), but could also use multiple fluors in a single track. Thus if (for example) four sets of each of 100 probes were assembled, the probes of each set flanked by different primer binding sites (same within a set), they could be hybridised with the same target, and after the initial recovery of bound probes and low-level preamplification using all primer pairs, each set could be selectively amplified with its own primer pair (one fluorescent), and the four resulting sets of fluorescent products mixed and run in the same lane. Similarly, if the same primer was available with different fluorescent labels, then a direct comparison between two samples (for example tumour versus normal from the same person) could be made by labelling the products from each with different fluors and running in the same lane of the gel.

In the present format, the high resolution of polyacrylamide gel electrophoresis allows the simultaneous differentiation of products from many different loci, but even using all the available "slots" with judicious assembly of probe sets, this format probably limits the number of probes flanked by a given primer pair which can be resolved in one lane to about 500, and hence if four probe sets with different flanking primers can be used simultaneously, fewer than 2000 probes can be examined simultaneously in this test. It might be possible to augment the resolution of this analysis by using formats for the quantitative detection of amplified probe which do not rely on gel electrophoresis. One alternative would be to use recovered probes to label

arrays of the clones from which the probes themselves were produced; a procedure analogous to array-CGH (Pinkel et al., 1998), but with the added efficiency that the method according to the present invention the MAPH has already produced proportionate amounts of the relevant single-copy probes from the genomic targets, reducing the complexity of the mixture (for increased speed of hybridisation) and by avoiding multi-copy sequences, presumably allowing quantification with a greater signal:noise ratio.

The experiments explained here have used genomic DNA as the target nucleic acid, but an extension of this method would be to use probes to probe RNA (or cDNA) for highly parallel analysis of gene expression. Current RT-PCR based methods for quantification of gene expression do not allow the simple analysis of numerous loci simultaneously.

The method could be used to screen for alterations to a plurality of sequences relating to different genes, and/or sequences within a target gene e.g. screen for alterations in exon copy number within a eukaryotic gene.

Using the method according to the present invention to detect deletions and amplifications in (for example) idiopathic mental retardation (IMR) assumes that the absence of a locus is abnormal, rather than resulting from a polymorphism. This can be most simply investigated by analysis of the parents in cases of IMR, but it remains possible that wider application of this technique will uncover loci at which there are presence/absence polymorphisms. If enough (sufficiently informative) loci of this kind were available, then assembly of such probes into a mutually compatible "polymorphic set" would allow simultaneous genotyping at (say) 50-100 loci in a single lane of a sequencing gel; combined with analysis of mixed DNA samples, such a system could provide very high throughput for genotyping without the need for investment in expensive or complicated equipment.

Whilst endeavouring in the foregoing specification to draw attention to those features of the invention believed to be of particular importance it should

be understood that the Applicant claims protection in respect of any patentable feature or combination of features hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.

CLAIMS

1. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material (10), the method comprising introducing to the sample (10) a plurality of different genetic probes (14) suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample (10) to conditions favouring hybridisation of the probes (14) to their respective sequences, and amplification of sample-bound probes (14) using a pair of primers, wherein analysis of the respective amounts of amplified probe (14) provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample (10).
2. A method according to claim 1, characterised in that each probe (14) is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel.
3. A method according to claim 1 or 2, characterised in that the plurality of different probes (14) comprises a predetermined set of different probes (14) each chosen to be specific for a respective target nucleic acid sequence.
4. A method according to claim 3, characterised in that the set comprises probes (14) suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced enables quantitative determination of the copy number of the respective sequences in the sample.
5. A method according to claim 4, characterised in that the polymerase chain reaction is used to determine the quantity of each probe product produced.
6. A method according to any of the preceding claims, characterised in that the method is used to screen sequences of different genes or different

sequences within a gene, such as different exons in a eukaryotic gene.

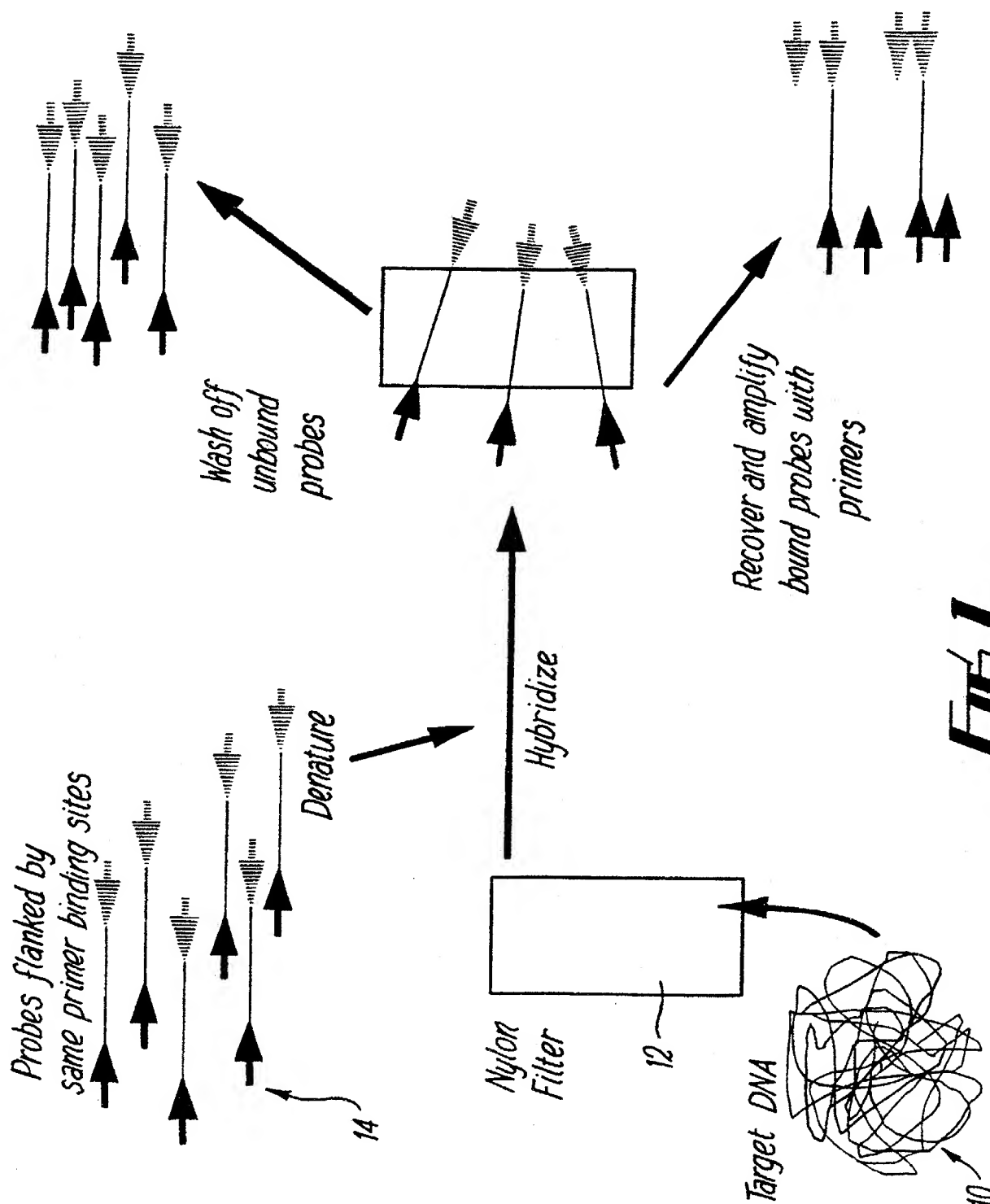
7. A method according to any of the preceding claims, characterised in that the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).
8. A method according to any of the preceding claims, characterised in that the genetic material (10) is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised.
9. A method according to any of the preceding claims, characterised in that an excess of probes (14) is used.
10. A method according to any of the preceding claims, characterised in that probes (14) labelled for ready identification are used.
11. A method according to claim 10, characterised in that probes labelled with fluorescent labels are used.
12. A method according to any of claims 3 to 11, characterised in that more than one set of probes (14) is used, either simultaneously or sequentially.
13. A method according to claim 12, characterised in that the flanking primer pairs are the same for each set of probes (14).
14. A method according to claim 12, characterised in that the flanking primer pairs are different for each set of probes (14).
15. A method according to any of the preceding claims, characterised in that the method comprises means to obviate or mitigate hybridisation between primer binding sequences.



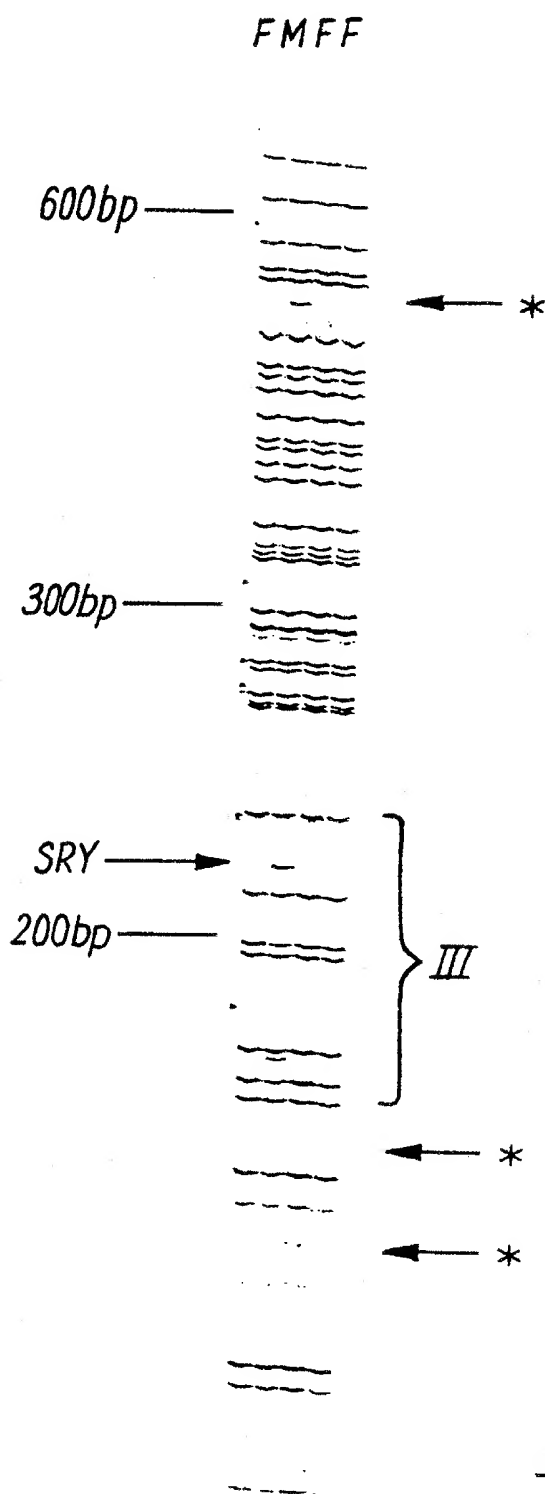
16. A method according to claim 15, characterised in that competing oligonucleotides are introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.
17. A method according to any of the preceding claims, characterised in that unbound probes (14) and primers are thoroughly washed away from the bound probes (14) following hybridisation stage and prior to analysis.
18. A method according to any of claims 3 to 17, characterised in that the method is used to screen DNA, RNA and/or cDNA with appropriate probe sets.
19. A method according to claim 18, characterised in that the method is used to screen somatic and/or germline sequences.
20. A method according to claim 18 or 19, characterised in that the method is used to screen for polymorphic alterations.
21. A set of probes (14) substantially as described above in any of the preceding claims.
22. A kit for such a method described above in any of the preceding claims, which kit comprises a probe set (14) generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).
23. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.
24. A set of probes substantially as hereinbefore described with reference to the accompanying examples and drawings.

25. A kit for a method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.

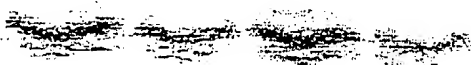
26. Any novel subject matter or combination including novel subject matter disclosed herein, whether or not within the scope of or relating to the same invention as any of the preceding claims.

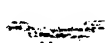


**FIG. 1**

**FIG. 2**

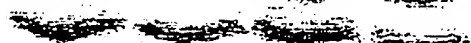
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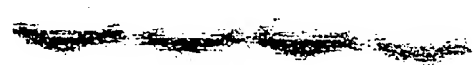
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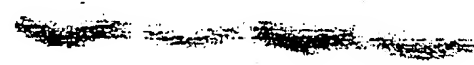
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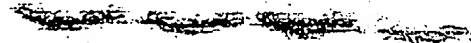
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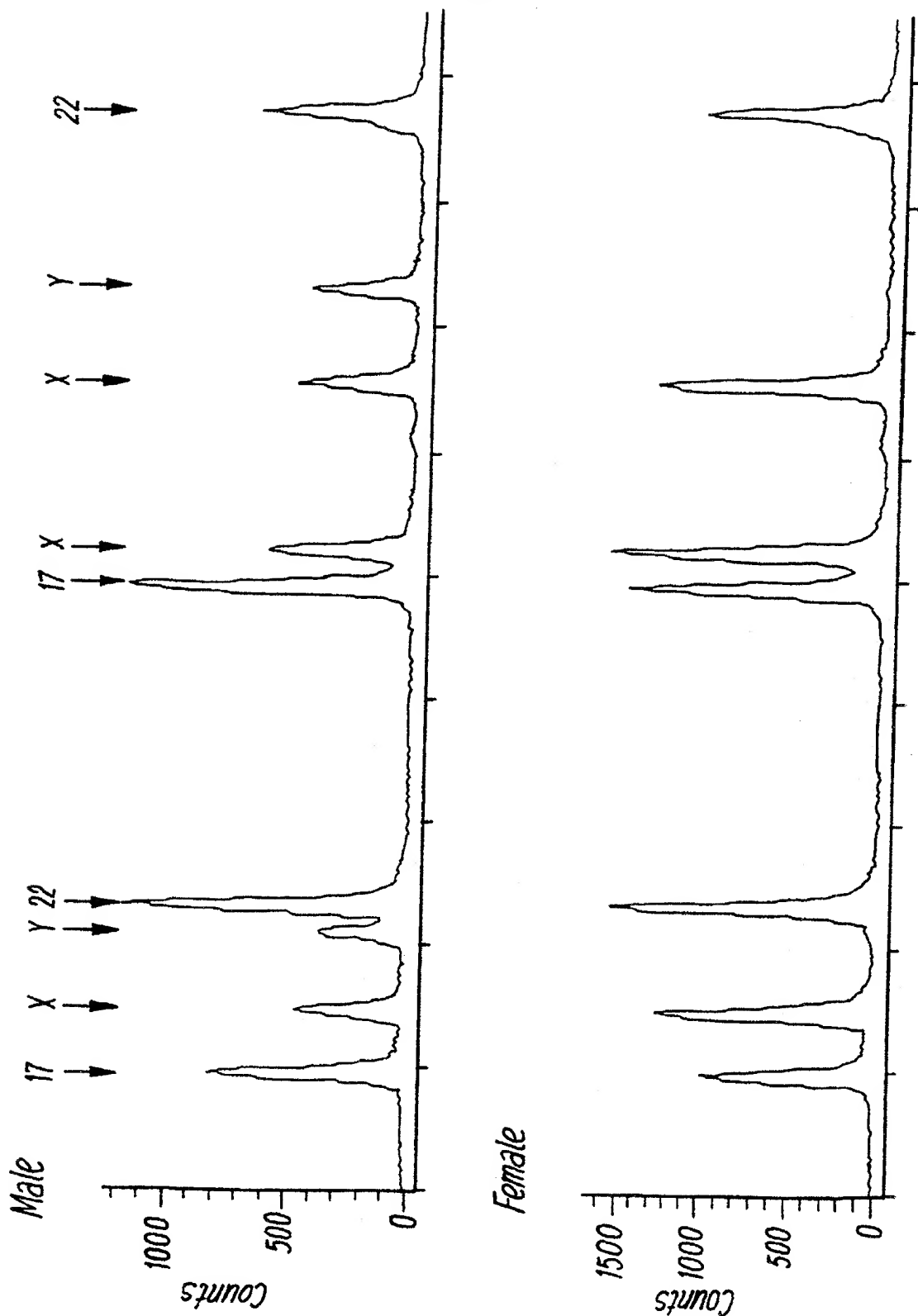
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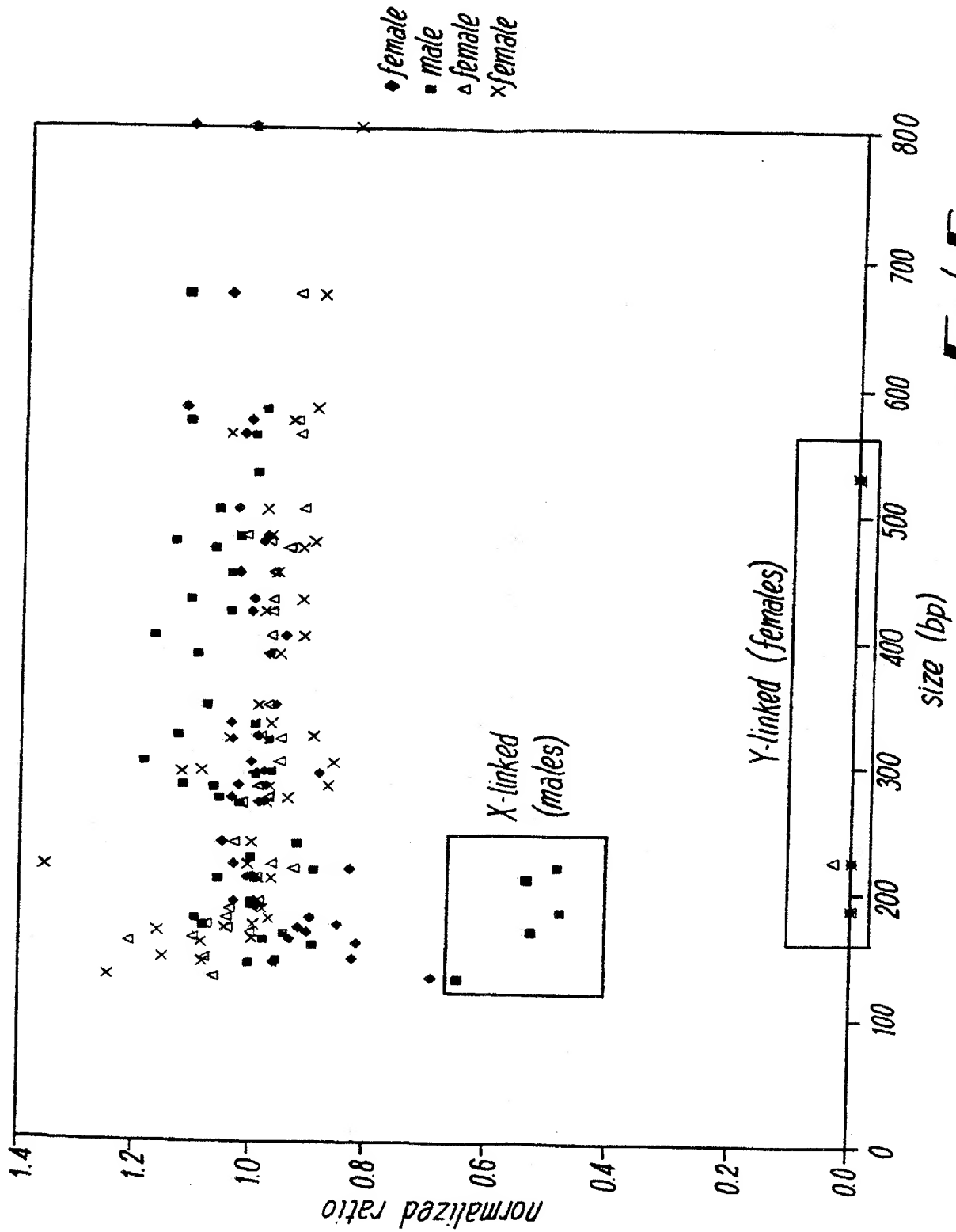
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17 → 

**FIG. 3**



**Fig 4**



**Fig. 5**

primer PZA  
 AGTAACGGCCGCCAGTGTGCTG EcoRV  
 GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGatatccATCACACTGGCGGCCGCTCGAGCA  
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+  
 CCTAGGTGATCATTCGCCGGGTCACACGACCTTAAGACGTctatagTAGTGTGACCGCCGGCGAGCTCGT  
 GTAGTGTGACCGCCGGCGAGC  
 primer PZB

Fig. 6